Detection of Enterotoxigenic *Clostridium perfringens* in Raw Beef by Polymerase Chain Reaction

ABSTRACT

A polymerase chain reaction (PCR) procedure was developed for direct detection of *Clostridium perfringens* strains with potential for food poisoning in raw beef samples. An oligonucleotide primer pair was used to amplify a 364 base pair sequence internal to the *C. perfringens* enterotoxin gene. One milliliter portions of the meat homogenates were inoculated into cooked meat medium (CMM) or reduced Fluid Thioglycollate (FTG) medium and incubated at 37°C. Portions sampled at 2, 4, 6, 8 and 24 h of enrichment were assayed for detection of the enterotoxin sequence by PCR. Amplification of the 364 bp sequence could be detected in 6 h by agarose gel electrophoresis and as early as 2 h by hybridization to a 150 bp digoxigenin (DIG)-labeled probe. To increase the sensitivity of the detection assay a commercial chromosomal deoxyribonucleic acid (DNA) extraction assay was compared with a nested PCR approach. Both methods allowed detection of less than 1 $\log_{10}$ colony forming units (CFU)/g of *C. perfringens* strains harboring the enterotoxin gene, with no interference with the background microflora present in the raw ground beef.

Key words: *Clostridium perfringens*, enterotoxin gene, polymerase chain reaction

Clostridium perfringens type A, considered one of the most common human food borne pathogens in the United States, is acquired through the consumption of contaminated meat and poultry products (*21*). Presently, identification of *C. perfringens* from food samples requires a lengthy biochemical characterization through the use of selective enrichment followed by biochemical tests and final confirmation of enterotoxigenic strains by serological analysis (*11*). A number of identification approaches for the detection of enterotoxigenic *C. perfringens* from food samples following cultivation and isolation of the target organism have been previously reported (*2,16,19,30,33*). These approaches require a long time to provide a positive identification, whereas rapid results are of crucial importance when dealing with perishable foods or in cases of food poisoning outbreaks.

Among current molecular detection techniques, the PCR is finding increasing applications in diagnostic food microbiology by providing specific and sensitive identification of a number of food pathogens. However, the application of this powerful technique has met difficulties inherent to the complex nature of different food matrices. The latter has resulted in decreased sensitivity of the technique associated with the inhibition of the Taq polymerase (*25,35-37*). Several researchers have optimized and adapted the PCR technique to diagnostic food microbiology. Modifications include combinations of centrifugation and filtration of food homogenates (*35*), DNA extraction and purification (*1,7,8,10,14,18,36*), immunomagnetic separation (*5,27*), and the use of pre-enrichments for amplification of the number of cells and, therefore, target sequence(s) (*1,7,10,17,23*).

To our knowledge, the direct PCR testing of meat samples for the presence of enterotoxigenic *C. perfringens* has not been reported previously. In this study, we compared media enrichments and amplification parameters necessary to detect enterotoxigenic *C. perfringens* in raw ground beef using a PCR procedure.

MATERIALS AND METHODS

Bacterial strains

Clostridium perfringens strains NCTC 8238, NCTC 8239 and ATCC 10288, obtained from our culture collection, were used for enterotoxin detection. Non-enterotoxigenic *C. perfringens* strains ATCC 3624 and FD-1 were used as negative controls. The latter two strains were kindly provided by Dr. R. Labbe (University of Massachusetts, Amherst, MA). The potential for enterotoxin production and the presence of the enterotoxin gene were confirmed by a reversed passive latex agglutination assay (SPET-RPLA) (Oxoid, Inc., Columbia, MD) and by PCR amplification, respectively. In the latter, the oligonucleotide primer pair CPEPS/CPENS, shown in Table 1, was used for amplification of the 364 bp enterotoxin fragment (data not shown). Stock cultures were maintained in cooked-meat medium (CMM; Difco Laboratories, Inc., Detroit, MI), and stored at 4°C throughout the course of the study. Vegetative cell cultures were grown by inoculating 0.1 ml of the stock culture into 10 ml of freshly prepared FTG medium (Difco). The inoculated medium was heat-shocked at 75°C for 20 min followed by aerobic overnight incubation at 37°C. For determination of the number of vegetative cells, the cultures were serially diluted in 0.1% (wt/vol) peptone-water and

TABLE 1. *Synthetic oligonucleotide sequences.*

Oligonucleotide	Sequence[a] (5' to 3')	Location on CPE gene
CPEPS	TGTAGAATATGGATTTGGAAT	426 - 446
CPENS	AGCTGGGTTTGAGTTTAATGC	789 - 769
INTPS	CAAATGAATATGTATATTATAA	500 - 522
INTNS	ATATTTCCTAAGCTATCTGCAG	650 - 629

[a] Reference No. 32.

plated onto tryptose-sulfite-cycloserine (TSC) agar overlaid with an additional 10 ml of TSC agar (*13*). The plates were incubated overnight at 37°C in anaerobic jars (BBL GasPack Anaerobic Systems, Beckton Dickinson, Cockeysville, MD).

Meat preparation and enrichment procedure

Ground beef was obtained from local retail markets. Individual portions of 20 g were aseptically weighted into filter Stomacher™ bags (SFB-0410; Sprial Biotechnology, Bethesda, MD) and inoculated with 1 ml of the appropriate dilution of *C. perfringens* cell suspension to obtain between 0 and 4 $\log_{10}$ CFU/g. Negative controls consisted of either meat samples inoculated with 1 ml of 0.1% (wt/vol) peptone-water with no bacterial cells or the enterotoxin-negative strains ATCC 3624 or FD-1. Each sample was diluted with 20 ml of filter-sterilized phosphate buffered saline (PBS, pH 7.4) containing 0.1% Tween 80 (Sigma Chemical Co., St. Louis, MO). The beef samples were homogenized for 1 min in a Stomacher™ Lab-Blender 400 (Tekmar Company, Cincinnati, OH). Meat homogenates (7 to 10 ml) were transferred to sterile 15 ml conical screw cap tubes (Sarstedt, Inc., Princeton, NJ) and centrifuged at 500 rpm for 5 min in a Sorvall GLC-1 centrifuge (Du Pont Company, Wilmington, DE). One milliliter of the supernate was transferred to 9 ml of reduced FTG medium and CMM and incubated at 37°C. To estimate the minimal incubation period required to detect the enterotoxin gene, 1.0 ml and 0.1 ml samples were withdrawn at 0, 2, 4, 6, 8 and 24 h. The 1.0 ml portions were collected for extraction of total chromosomal DNA before the PCR amplification step using a commercial G-NOME™ DNA isolation kit (Bio 101, Inc., La Jolla, CA). The 0.1 ml portions were used for direct PCR detection by boiling the sample for 5 min followed by centrifugation at 12,000 rpm for 5 min.

Bacterial enumeration

The cell numbers for the enterotoxin positive and enterotoxin negative strains were determined by plating serial dilutions of the corresponding meat supernatant fluid onto TSC agar. As described above, the diluted samples were plated in duplicate on TSC agar using a spiral plater (Spiral), and also by inoculating 0.1 ml of the meat supernatant fluid onto TSC agar. The plates were overlaid with an additional 10 ml of TSC agar and incubated overnight at 37°C in anaerobic jars.

Background bacterial flora present in raw ground beef was also enumerated for CFU. Uninoculated meat supernatant fluids were serially diluted and plated in duplicate onto brain heart infusion (BHI) agar, and plate count agar (Difco), using a spiral plater. Duplicate plates were incubated aerobically at 37°C and 42°C for 24 h.

Primers

Oligonucleotide sequences and the corresponding positions in the *C. perfringens* enterotoxin gene are shown in Table 1. The oligonucleotide sequences CPEPS and CPENS were previously reported by Saito, Matsumoto and Funabashi (*26*). All the primers were synthesized by beta cyanoethyl phosphoramidite chemistry (Appligene, Inc., Pleasanton, CA).

PCR amplification

Amplification reactions were performed in 50 µl volumes. The reaction mixture contained 3 µl of the test sample (extracted chromosomal DNA or boiled sample), 200 µM concentrations of dATP, dCTP, dGTP and dTTP, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.5 µM of each primer (CPEPS/CPENS), 1.5 mM $MgCl_2$, 0.01% gelatin and 1.25 units *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, CT). After overlaying with mineral oil, the samples were subjected to 30 cycles of amplification in a PTC-100™ Programmable Thermal Controller (MJ Research, Inc., Watertown, MA).

Two different amplification conditions were initially assayed using chromosomal DNA purified by the method described by Saito, Matsumoto and Funabashi (*26*). In the first, a standard-high temperature cycling routine (*15*) consisted of denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and extension at 74°C for 2 min, with a cycle extension of 3 s/cycle. In the second, a shorter incubation-low temperature cycling routine for amplification of clostridial DNA was used (*31*). After 30 amplification cycles, both temperature cycling routines terminated with a final extension incubation by holding the tubes at 74°C for 10 min and then cooling to 4°C. The samples were precipitated with two volumes of ethanol, resuspended in 20 to 30 µl of water, and 50% of the samples were subjected to electrophoresis in a 2% agarose gel. The gels were stained with ethidium bromide (0.5 µg/ml) for 15 to 20 min (*20*), and the amplification products were visualized using a FOTO/PREP I transilluminator (Fotodyne, Inc., New Berlin, WI).

To increase the sensitivity of the *C. perfringens* enterotoxin gene detection, a nested PCR was done using 10 µl of the first PCR amplification in a second round of PCR containing a primer set internal to the 364 bp fragment (INTPS/INTNS) (Table 1). The nested amplification reaction was subjected to 30 automated cycles using the high temperature cycling routine described above.

Clostridium perfringens *enterotoxin gene probe (CPE)*

A 150 bp DIG-labeled DNA probe was generated by incorporation of the DIG-dUTP in the PCR amplification reaction. PCR reactions were set up in a final volume of 100 µl containing 200 µM concentrations of dATP, dCTP and dGTP; 167 µM dTTP, 33 µM digoxigenin-11-dUTP (Genius™ Systems, Boehringer Mannheim Biochemicals, Indianapolis, IN), 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% gelatin, 2.5 units *Taq* polymerase (Perkin-Elmer Cetus), 0.5 µM of each primer (INTPS/INTNS), and 500 to 850 ng template genomic DNA (NCTC 8239 strain) purified by the method of Saito, Matsumoto and Funabashi (*26*). Template DNA was denatured at 100°C for 10 min and placed on ice immediately before addition to the PCR reaction. Thirty cycles of PCR amplification were run using the higher temperature cycling conditions described above. The amplified products were detected by electrophoresing 10 to 15 µl of the reaction mixture through a 2% agarose gel in 40 mM Tris-acetate, 1 mM ethylenediaminetetraacetic acid (EDTA) (TAE:pH 8.0) followed by ethidium bromide staining (*20*). The concentration of the amplified products was also measured by absorbance at 260 nm (A_{260}).

Probe hybridization and detection PCR products

The electrophoresed PCR products were transferred onto Nylon 66 Plus membranes (Hoefer Scientific Instruments, San Francisco, CA), by the method of Southern (*28*), and baked at 80°C for 2 h in a conventional oven to fix the DNA. The membranes were then transferred to 50 ml conical centrifuge tubes (Sarsted, Inc., Princeton, NJ) and hybridized to an internal

150 bp DIG-labeled probe at a final concentration of 20 ng/ml. The hybridization reaction was incubated overnight at 65°C. Next, the membranes were transferred to 15 × 100 mm petri plates (Fisher Scientific Co., Malvern, PA) for colorimetric development of the hybridization reaction using a DIG-DNA detection kit according to the manufacturer's instructions (Genius). The substrate solution was pre-incubated at 37°C before development of the color reaction. The damp membranes were immersed in the substrate solution and incubated at 37°C in the dark with no agitation. Color development was monitored for 15 to 30 min. The reaction was stopped by washing the membranes with distilled water followed by a Tris-EDTA solution (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The stained membranes were stored in the dark.

RESULTS

A 364 base pair sequence, previously reported by Saito, Matsumoto and Funabashi (*26*), was selected as target for PCR detection. The experimental conditions for PCR amplification of the 364 bp enterotoxin fragment were first assessed with purified template DNA. The lower temperature cycling conditions allowed for an amplification time of approximately 2.5 h, due to shorter denaturation and annealing times, whereas the higher temperature cycling conditions required approximately 4 h for completion. The high-temperature amplification resulted in a tenfold greater sensitivity as observed by ethidium bromide-stained agarose gel electrophoresis. This high-temperature cycling routine was chosen for the detection of the enterotoxin sequence from the meat homogenates and enrichments. The experiments were repeated twice for each enterotoxigenic *C. perfringens* strain and the results were consistent through the study.

Meat supernatant fluids obtained after homogenization were centrifuged at low speed to sediment large particulates before sampling or inoculation into enrichment media. The supernatant fluids were also used for anaerobic plate count (APC) to enumerate CFU of *C. perfringens* in the spiked beef samples. Polymerase chain reaction was performed on meat homogenates spiked with 10^{-2}, 10^{-4}, 10^{-5}, 10^{-6} dilutions of the overnight cultures which corresponded to 4, 2, 1 and 0 $\log_{10}$ CFU per gram.

Direct PCR amplification of the 364 bp enterotoxin sequence was performed with the 0.1 ml samples after boiling and centrifugation. The amplified product could be visualized by ethidium bromide-stained 2% agarose gel as early as 6 h of incubation for the CMM and FTG medium (Fig. 1). Amplification signals corresponding to levels below 1 $\log_{10}$ CFU/g of *C. perfringens* could be detected in the CMM enrichments whereas the FTG showed a tenfold lower sensitivity (2 $\log_{10}$ CFU/g) at the 6-h incubation. Enrichment for 8 h or more resulted in efficient amplification and detection below 1 $\log_{10}$ CFU/g.

Confirmation of the amplified PCR products was performed by hybridization to an internal DNA probe. The oligonucleotide primer sequences (INTPS/INTNS) shown in Table 1 were used to generate a 150 bp DIG-labeled probe internal to the *C. perfringens* 364 bp PCR product. The sensitivity of the hybridization technique allowed for detection of the amplification products as early as 2 h in the enrichment incubation as shown in Fig. 1. No amplification signal was detected immediately after dilution in enrichment medium (T_0) or when undiluted meat supernatants were assayed. The negative controls, which included uninoculated beef samples containing a bacterial background flora of approximately 6 $\log_{10}$ CFU/g, and beef samples inoculated with enterotoxin negative strains, showed no reaction as confirmed by the absence of a hybridization signal (data not shown).

A commercial chromosomal DNA extraction kit and a nested PCR protocol were used to increase the sensitivity of the detection assay for the shortest enrichment time (2 h). Efficient amplification was achieved when the total DNA present in the samples was extracted and reconstituted to a 10-fold of the original 1 ml sample (100 µl final volume). Figure 2A shows the amplification products after the extraction of total chromosomal DNA obtained after the 2-h enrichment incubation. This method resulted in an increased sensitivity of detection compared to direct boiling of the samples (Fig. 3A). This was evidenced by the detection of amplification products by agarose gel electrophoresis only when the samples were subjected to the DNA extraction procedure before PCR.

Nested PCR of both reaction products (boiled and extracted) confirmed the amplification of the 364 bp sequence as demonstrated by the internal 150 bp product (Fig. 2B and 3B). Both procedures (nested PCR and chromosomal DNA extraction) detected <1 $\log_{10}$ CFU/g of *C. perfringens*. The chromosomal extraction procedure involved a maximum of 2 h of sample processing prior to the PCR amplification. On the other hand, the nested PCR approach required an additional 4 h after the first round of PCR amplification.

Samples processed by either method, boiled or extracted, showed a non-specific amplification product of (~500 bp) after the nested PCR with the INTPS/INTNS primer pair. The non-specific product did not hybridize to the 150 bp DIG-labeled probe as shown in Fig. 2B and 3B.

DISCUSSION

Confirmation of enterotoxigenic *C. perfringens* strains by conventional reverse passive latex agglutination has proven difficult for strains that show poor sporulation in culture media and may require up to 72 h (*3,12,24*). The frequent association of *C. perfringens* with outbreaks of food poisoning where meat and poultry products are consumed (*9,21*) warrants the development of a detection assay for these type of food matrices while providing rapid identification in a sensitive and specific fashion.

In the present study, detection of enterotoxigenic *C. perfringens* in raw beef was achieved by nucleic acid amplification of a portion of the enterotoxin gene. The target gene was selected because of its association with pathogenicity in food poisoning outbreaks (*4,6,22,29,34*). This approach specifically amplifies the enterotoxin A gene, excluding any non-enterotoxigenic strains. This is in contrast to methods based on detection of highly conserved ribosomal sequences.

Detection of the enterotoxin gene was accomplished by a combination of a short incubation and a nucleic acid

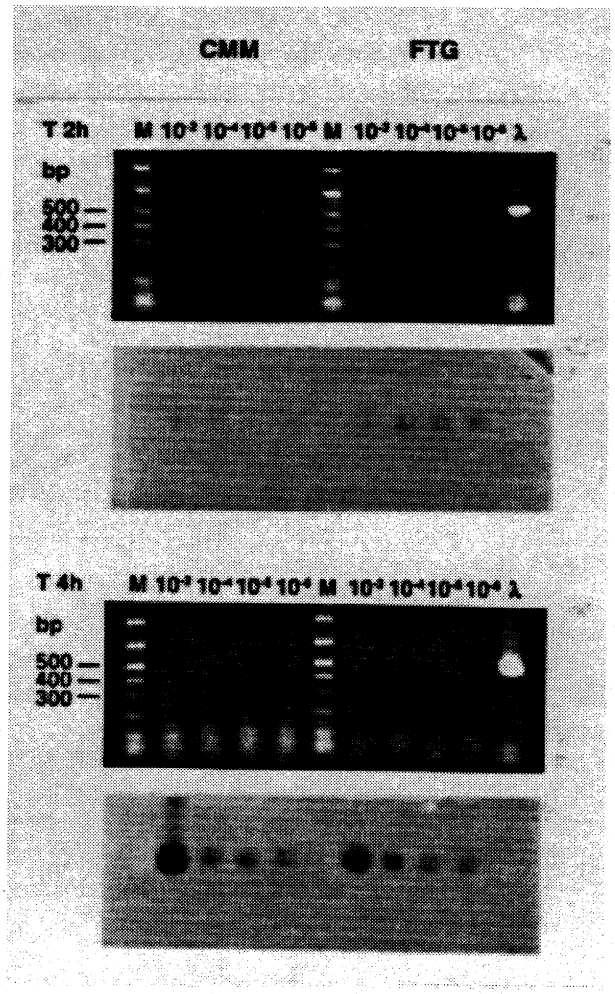

Figure 1. *Agarose gel electrophoresis and Southern blot analysis of PCR amplified products after 24-, 8-, 6-, 4- and 2-h enrichment in CMM and FTG. Primer set CPEPS/CPENS was used for amplification of a 364 bp sequence of the enterotoxin gene. The panel shown is representative of the amplification results after meat samples were spiked with 10^{-2}, 10^{-4}, 10^{-5} and 10^{-6} dilutions of overnight cultures of enterotoxigenic* C. perfringens. *The meat supernatant fluids contained between 2.15 × 10^{6} to 8.00 × 10^{6} CFU/g of C. perfringens as determined by APC. M: BioMarker Low™ molecular weight marker. λ: Bacteriophage Lambda DNA amplification control.*

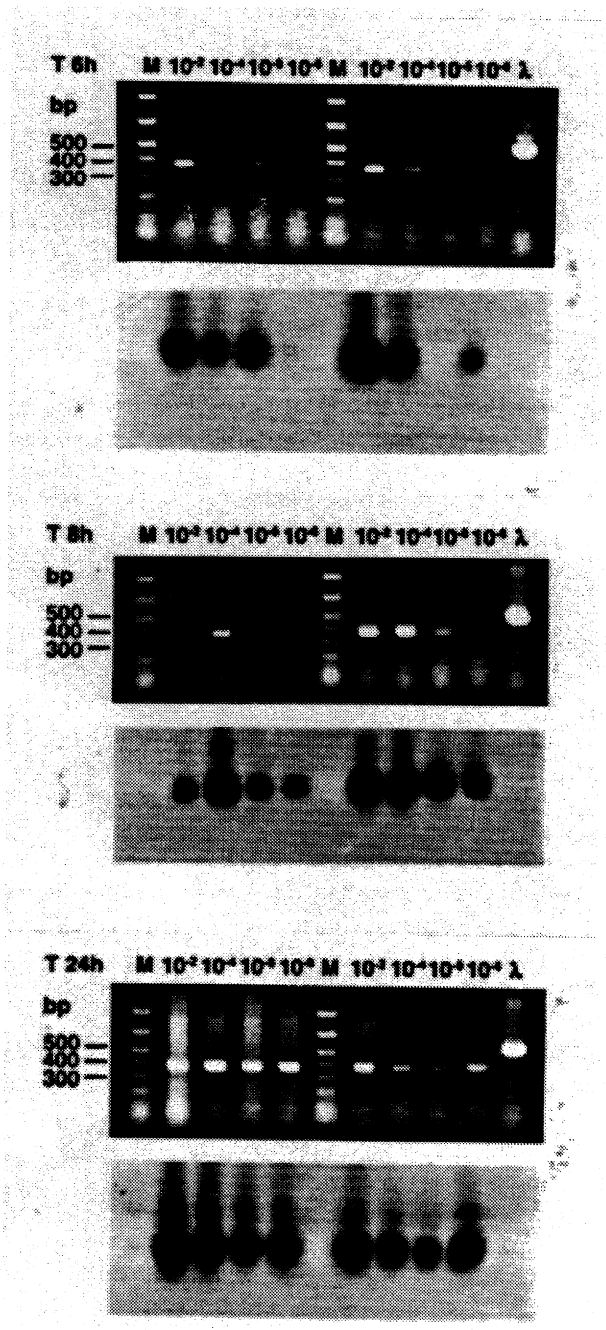

Figure 1. *Continued.*

extraction step for the enriched portion. The enrichment increased the number of cells and, therefore, the number of target sequences thus improving the final sensitivity of the assay, and reducing the inhibitors of the PCR assay potentially present in the undiluted meat homogenate (*25*).

Confirmation of the PCR-amplified products was accomplished by Southern hybridization with an internal-nonisotopic probe. Labeling of the 150-base pair DNA probe was performed by incorporation of the DIG-dUTP during a PCR amplification. When compared to oligonucleotide terminal labeling, this simple labeling procedure provided large amounts of the DIG-labeled gene probe containing a higher signal ratio, due to a more frequent incorporation of the DIG-labeled dUTP. This method of labeling provided a superior probe sensitivity (estimated around 0.2 pg) for the detection assay. The sensitivity provided by the gene probe allowed for the detection of an amplification signal within the 2-h enrichment incubation.

To improve the sensitivity of the assay, the nested PCR approach was compared to a chromosomal DNA extraction step for the 2-h enrichment incubation. While the former

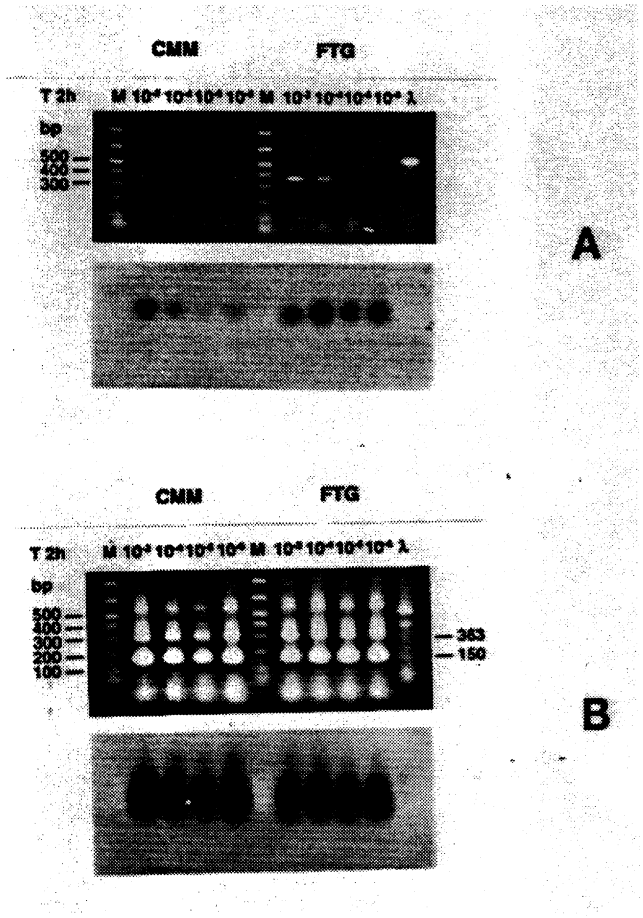

Figure 2. *Agarose gel electrophoresis and Southern blot analysis of PCR amplified products after 2-h enrichment in CMM and FTG. Total chromosomal DNA was extracted from 1 ml samples before portions of 3 µl were assayed by PCR. (A): PCR amplification products after 30 cycles, and corresponding blot against the DIG-labeled 150 bp internal probe. (B): Nested PCR amplification using the INTPS/INTNS primer pair and corresponding blot against the homologous 150 bp DIG-labeled probe. M: BioMarker Low™ molecular weight marker. λ: Bacteriophage Lambda DNA amplification control.*

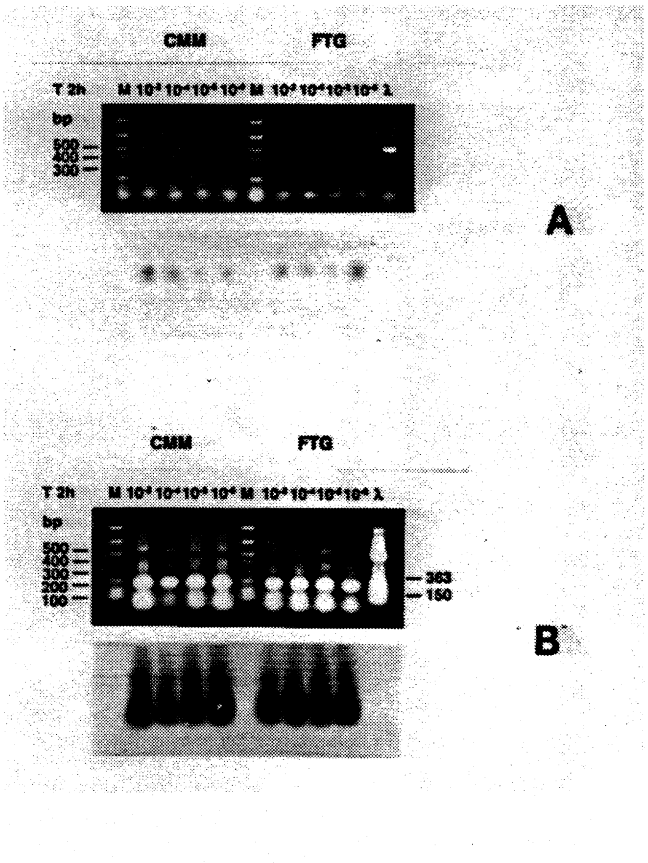

Figure 3. *Agarose gel electrophoresis and Southern blot analysis of PCR amplified products after 2-h enrichment in CMM and FTG. 0.1 ml samples were boiled at 100°C for 5 min and centrifuged at 12,000 rpm for another 5 min before portions of 3 µl were assayed by PCR. (A): PCR amplification products after 30 cycles, and corresponding blot against the DIG-labeled 150 bp internal probe. (B): Nested PCR amplification using the INTPS/INTNS primer pair and corresponding blot against the homologous 150 bp DIG-labeled probe. M: BioMarker Low™ molecular weight marker. λ: Bacteriophage Lambda DNA amplification control.*

required an additional 4 h before the amplification products could be detected, the latter required no more than 2 h of sample processing before PCR. Amplification signals at levels below 1 $\log_{10}$ CFU/g were consistently detected by either, the nested PCR or the chromosomal DNA extraction. However, incorporating the chromosomal DNA extraction prior to the PCR amplification yielded a concentrated and purified template for the PCR reaction within a shorter assay time.

In conclusion, the PCR amplification assay developed in this study combined a short enrichment incubation with a chromosomal DNA extraction step preceding amplification. Pre-enrichment assured detection of only viable organisms in the food product. The added nucleic acid extraction step provided a purified target DNA within a heterogeneous DNA pool present in the extracted portion. Upon dilution, this template DNA constituted an ideal sample for PCR amplification as reflected by the superior amplification observed by agarose gel electrophoresis.

The detection of *C. perfringens* in spiked beef samples demonstrated a higher level of sensitivity over conventional cultivation methods, while providing direct evidence for the presence of *C. perfringens* strains harboring the enterotoxin gene. The assay detected the clostridial enterotoxin A gene at levels below 1 $\log_{10}$ CFU/g of meat in the presence of the background flora (~10^6 CFU/g) present in raw beef.

Overall, the assay required 2 h of enrichment incubation, followed by a 2-h sample extraction step, and finally 4 h of PCR amplification. The entire sample analysis required over 8 h to complete when sample preparation before enrichment and the final detection step are taken into consideration. The technique holds promise, due to its capability of handling large number of samples, its specificity and its sensitivity. Current efforts in our laboratory are aimed at assessing improvements in oligonucleotide primer selection for shortened amplification time and the application of faster detection procedures for the amplified products.

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